Monatsh Chem (2009) 140:121–127
DOI 10.1007/s00706-008-0052-z

ORIGINAL PAPER

Toward the synthesis and biological evaluation of hirsutide

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Received: 24 July 2008 / Accepted: 30 July 2008 / Published online: 14 October 2008

Abstract The present investigation deals with the synthesis of a *N*-methylated cyclotetrapeptide, hirsutide (**2**), by coupling of the dipeptide units Boc-L-phenylalanyl-L-*N*-methylphenylalanine-OH and L-valyl-L-*N*-methylphenylalanine-OMe followed by cyclization of the linear tetrapeptide fragment. The chemical structure was established on the basis of analytical as well as spectroscopic data. The newly synthesized cyclic peptide was subjected to pharmacological screening and found to be highly potent against the gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* at 6 μg cm^{-3}. In addition, potent antihelmintic activity against the earthworms *Megascoplex konkanensis* and *Pontoscotex corethruses* at 1 and 2 mg cm^{-3}, and potent cytotoxic activity against *Dalton's lymphoma ascites* and *Ehrlich's ascites carcinoma* cell lines with IC_{50} values of 14 and 22 μM were also observed. Studies revealed that the pentafluorophenyl ester method employing a catalytic amount of *N*-methylmorpholine proved to be better for cyclization of the linear tetrapeptide unit.

Keywords Natural products · Hirsutide · Peptides · Cyclizations · Total synthesis · Biological activity

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Introduction

Literature is enriched with several findings suggesting the vital role of natural products in pharmaceutical research as biomedically useful agents or as lead compounds for drug development. Among these, cyclopeptides and related congeners having unique structures and wide pharmacological profiles have emerged as an important class of organic compounds, which may prove better candidates to overcome the problem of resistance towards conventional agents [1–3]. Fungi-derived natural cyclic peptides exhibit a variety of bioactivities, such as cytotoxic activity [4, 5], antimalarial activity [6], insecticidal activity [7], antibacterial activity [8], antimycotic activity [9], antidinoflagellate activity [10], antimycobacterial activity [11], and inhibitory activity toward the spore germination of fungi [12]. Hirsutide (**2**), a cyclotetrapeptide, has been isolated from the spider-derived entomopathogenic fungus *Hirsutella* sp. using semipreparative HPLC under isocratic conditions [13] and is unique in having two *N*-methylated phenylalanine units in its structure. Having only minute quantities of this cyclopeptide obtained from natural resources has restricted scientists from investigating its biological profile in detail. Further, the widespread increase of resistance against conventional antibiotics encourages the development of novel bioactive congeners with unexploited mechanisms of action.

Hence, keeping in view the biological potential of extracts of *Hirsutella* sp. and to obtain a bioactive peptide in good yield, the present work was aimed at the first total synthesis of the natural peptide hirsutide (**2**) using the solution-phase technique in a simple and economical manner. The study also includes testing of the peptide for its antibacterial, antifungal, antihelmintic, and cytotoxic effects.

Results and discussion

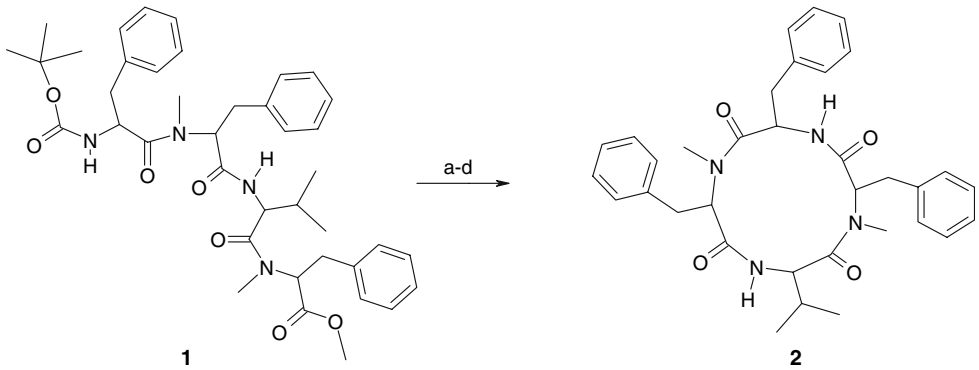
Chemistry

For the synthesis of hirsutide (**2**), the cyclic tetrapeptide molecule was disconnected into the two dipeptide units Boc-L-Phe-L-*N*-(Me)Phe-OMe and Boc-L-Val-L-*N*-(Me)Phe-OMe. The required amino acid methyl ester hydrochloride L-Phe-OMe.HCl and Boc-protected amino acids viz. Boc-L-Phe-OH and Boc-L-Val-OH were prepared according to previously reported procedures [14–17]. The free NH_2 group of L-Phe-OMe.HCl was protected by introduction of the Boc-group to get Boc-L-Phe-OMe. *N*-methylation of the Boc-protected phenylalanine methyl ester was achieved by treatment with methyl iodide and sodium hydride [18] to yield Boc-L-*N*-(Me)Phe-OMe. The Boc-group of the resulting unit was then removed using trifluoroacetic acid (TFA) to give L-*N*-(Me)Phe-OMe. The required dipeptide units were prepared by coupling of *N*-methyl-phenylalanine methyl ester with Boc-L-Phe-OH and Boc-L-Val-OH employing dicyclohexylcarbodiimide/*N*,*N*-diisopropylcarbodiimide/1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (DCC/DIPC/EDC.HCl) as the coupling agents and TEA as base [19–28]. Ester group of the dipeptide Boc-L-Phe-L-*N*-(Me)Phe-OMe was removed by alkaline hydrolysis with LiOH, and the deprotected peptide was coupled with the dipeptide Boc-L-Val-L-*N*-(Me)Phe-OMe after deprotection at the amino terminal, to get the linear tetrapeptide unit Boc-L-Phe-L-*N*-(Me)Phe-L-Val-L-*N*-(Me)Phe-OMe (**1**). The methyl ester group of the linear peptide fragment was then replaced by the *p*-nitrophenyl/pentafluorophenyl (pnp/pfp) ester group. The Boc-group of the resulting compound was removed using TFA, and the deprotected linear tetrapeptide fragment was now cyclized by keeping the whole contents at 0 °C for 7 days in the presence of a catalytic amount of NMM/TEA/pyridine to obtain cyclo(L-Phe-L-*N*-(Me)Phe-L-Val-L-*N*-(Me)Phe)—hirsutide (**2**) (Scheme 1).

The structures of the newly synthesized cyclic peptide as well as the linear tetrapeptide were confirmed by FTIR, ^{1}H-NMR, ^{13}C-NMR as well as elemental analysis. In addition, a mass spectra was recorded for the cyclotetrapeptide.

The synthesis of hirsutide (**2**) was accomplished with good yield utilizing three different carbodiimides. The pfp ester was more suitable than the pnp ester for cyclization of the linear tetrapeptide unit possibly due to the low reactivity of the pnp ester. Four signals between 5.16 and 5.75 ppm in the proton spectrum of **2** suggested a peptidic structure for the compound, with these signals being attributable to the alpha protons of four amino acid units. The structure of the newly synthesized cyclic tetrapeptide was found to be identical with natural hirsutide (**2**) as indicated by two broad singlets at 9.58 and 9.54 ppm corresponding to the imino protons of the phenylalanine and valine moieties in the ^{1}H NMR. The proton spectrum also showed signals for two *N*-methyl groups at 2.91 and 2.87 ppm, which are attached to the remaining two phenylalanine units. This fact was further supported by the presence of four signals due to carbonyl groups between 169.91 and 174.53 ppm in the ^{13}C NMR spectrum of **2**. The presence of two NH-protons and two *N*-methyl singlets in the proton spectrum of the tetrapeptide suggested a cyclic structure, which also is in accordance with the molecular formula of $C_{34}H_{40}N_4O_4$, derived from the FABMS. Presence of $(M + 1)^+$ ion peak at *m/z* 569.70 in mass spectrum of **2** [$(M + 1)^+$ at *m/z* 569.31 for natural hirsutide], along with other fragment ion peaks resulting from cleavage at 'Phe–*N*(Me)Phe' and '*N*(Me)Phe–Val' amide bond levels, showed the exact sequence of attachment of all the four amino acid moieties in a chain. Further, presence of immonium ion peaks at *m/z* 134.20 (*N*(Me)Phe), 120.20 (Phe) and 72.10 (Val) further

Scheme 1



a = LiOH, THF:H_2O (1:1), RT, 1 h; b = DCC, pfp/pnp, RT, 12 h;
c = TFA, $CHCl_3$, RT, 1 h; d = NMM/TEA/C_5H_5N, $CHCl_3$, 7 days, 0 °C.

confirmed presence of these amino acid moieties in the cyclopeptide structure. Moreover, appearance of peaks at *m/z* 422.60, 394.50, 261.40 and 233.30 indicated that the fragmentation also occurs at 'Val–*N*(Me)Phe' and '*N*(Me)Phe–Phe' amide bond levels to some extent. In addition, elemental analysis of **2** afforded values (with tolerance of ±0.03) strictly in accordance with the molecular composition (Fig. 1).

Analysis of cytotoxic activity data suggested that the cyclotetrapeptide **2** exhibited a high level of cytotoxic activity against DLA and EAC cell lines with IC_{50} values of 14 and 22 μM (7.81 and 12.57 μg cm^{-3}) in comparison to the standard drug—5-FU (IC_{50} values—37 and 91 μM) and the linear tetrapeptide **1** [IC_{50} values—21 and 25 μM (14.78 and 17.39 μg cm^{-3})]. Antihelmintic activity data revealed that **2** showed higher activity against *M. konkanensis* and *P. corethruses* at 1 and 2 mg cm^{-3}, in comparison to the standard drugs, albendazole and mebendazole. Antimicrobial activity data indicated that **2** exhibited a higher antibacterial activity than the reference drug, gatifloxacin, against the pathogenic gram-negative bacteria *P. aeruginosa* and *K. pneumoniae* at the 6 μg cm^{-3}. Furthermore, the synthesized peptide (**2**) showed moderate to good antifungal activity against the dermatophyte *M. audouinii* and the plant pathogenic fungus *Ganoderma* species, in comparison to the standard drug griseofulvin. However, **2** displayed only a moderate level of activity against the pathogenic fungi *C. albicans* and *A. niger*.

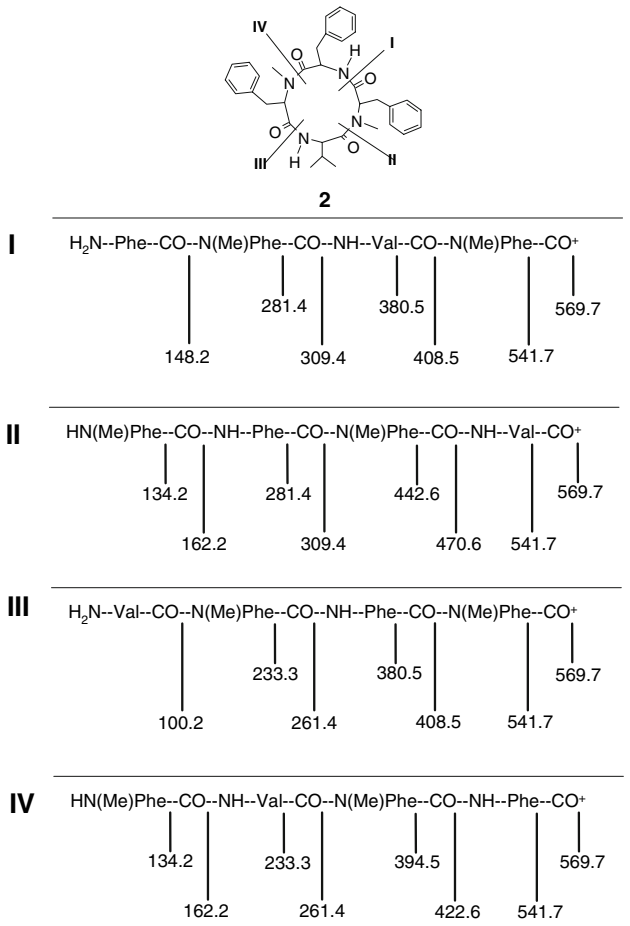


Fig. 1 Fragmentation pattern for hirsutide (**2**) in the mass spectrum

Analysis of pharmacological activity data revealed that the linear tetrapeptide **1** displayed less bioactivity against pathogenic microbes, earthworms and cell lines when compared to its cyclic form **2**. This is because cyclization of peptides reduces the degree of freedom for each constituent within the ring and thus substantially leads to reduced flexibility, increased potency, and selectivity of cyclic peptides. Further, the inherent flexibility of linear peptides leads to different conformations that can bind to more than one receptor, resulting in undesirable adverse effects. On passing toxicity tests, the synthesized cyclotetrapeptide **2** may prove a good candidate for clinical studies and can be a novel cytotoxic, antihelmintic, and antibacterial drug of the future.

In conclusion, the pentafluorophenyl ester was proved to be better for the activation of the acid functionality of the linear tetrapeptide unit. NMM was found to be a good base for intramolecular cyclization of the linear peptide fragment. DCC was found to be a yield-effective coupling agent giving a highly insoluble by-product dicyclohexylurea (DCU), in comparison to EDC.HCl and DIPC. A significant level of pharmacological potential was observed for the synthesized cyclotetrapeptide against gram-negative bacteria, earthworms, and DLA/EAC cell lines. Gram-positive bacteria and *Candida/Aspergillus* species were found to be the least sensitive to the cyclic peptide.

Experimental

Melting point was determined by open capillary method and is corrected. L-Amino acids, di-*tert*-butylpyrocarbonate (Boc_2O), DCC, TFA, TEA, NMM, and pyridine were purchased from SpectroChem Pvt. Ltd., Mumbai, India. EDC.HCl, DIPC, methyl iodide, sodium hydride, *p*-nitrophenol, and pentafluorophenol were procured from RANKEM RFCL Ltd., New Delhi, India. IR spectra were recorded on Shimadzu 8700 FTIR spectrophotometer (Shimadzu, Japan) using a thin film supported on KBr or $CHCl_3$ as solvent. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AC NMR spectrometer (300 MHz) (Brucker, USA) using $CDCl_3$ as solvent and TMS as internal standard. Mass spectra were recorded on a JMS-DX 303 Mass spectrometer (Jeol, Tokyo, Japan) operating at 70 eV using fast atom bombardment technique.

Elemental analyses of all compounds were performed on Vario EL III elemental analyzer (Elementar, Germany) and found to be in good agreement with the calculated values. Optical rotation was measured on automatic polarimeter (Optics Tech, Ghaziabad, India) in a 2-dm tube at 25 °C using sodium D-light. Purity of all compounds was checked by TLC on precoated silica gel G plates (Kieselgel 0.25 mm, 60G F254, Merck, Germany) utilizing $CHCl_3$/MeOH (9/1) and $CHCl_3$/AcOH/H_2O (3/2/5) as developing solvents.

Synthesis of linear tetrapeptide unit: Boc-L-Phe-L-N-(Me)Phe-L-Val-L-N-(Me)Phe-OMe (**1**, $C_{40}H_{52}N_4O_7$)

A solution of 2.92 g of L-Val-L-*N*-(Me)Phe-OMe (10 mmol) in 25 cm^3 of DMF was neutralized with 2.21 cm^3 of NMM (21 mmol) at 0 °C, and the resulting mixture was stirred for 15 min; 4.27 g of Boc-L-Phe-L-*N*-(Me)Phe-OH (10 mmol) was dissolved in 25 cm^3 of DMF and the resulting solution with 2.1 g of DCC [(10 mmol)/1.92 g of EDC.HCl (10 mmol)/1.26 g of DIPC (10 mmol)] was added to the above mixture. Stirring was first done for 1 h at 0–5 °C and then further for 24 h at RT. After the completion of the reaction, the reaction mixture was diluted with an equal amount of water. The precipitated solid was filtered, washed with water, and recrystallized from a mixture of chloroform and petroleum ether followed by cooling at 0 °C to get 5.9 g (84%) **1** as a semisolid mass. R_f 0.75 ($CHCl_3$:AcOH:H_2O/3:2:5); $[\alpha]_D^{20} = -81.6 \times 10^{-1}$ deg cm^2 g^{-1} (*c*, 0.9 in DMF); IR ($CHCl_3$): $\bar{\nu} = 3{,}222$–$3{,}217$, 3,112 (N–H str, amide), 2,968, 2,928–2,922 (C–H str, asym, CH_3 and CH_2), 2,877–2,873, 2,847 (C–H str, sym, CH_3 and CH_2), 1746 (C=O str, ester), 1,668–1,663, 1,639 (C=O str, amide), 1,589–1,585, 1,478–1,473 (skeletal bands, rings), 1,540–1,536 (N–H def, amide), 1,387, 1,368 (C–H def, butyl-*t*), 1,380, 1,364 (C–H bend, propyl-*i*), 1,269 (C–O str, ester), 715–705, 698–685 (C–H bend, out-of-plane, rings) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 9.05$ (brs, 1H, N*H*, Val), 7.50 (tt, 2H, $J = 7.15$, 4.35 Hz, H-*m*, Phe-1), 7.26 (tt, 2H, $J = 7.2$, 4.4 Hz, H-*m*, Phe-2), 7.14 (t, 1H, $J = 6.15$ Hz, H-*p*, Phe-3), 7.08–7.02 (m, 3H, H-*m*, Phe-3 and H-*p*, Phe-2), 6.89 (t, 1H, $J = 6.2$ Hz, H-*p*, Phe-1), 6.85 (dd, 2H, $J = 8.8$, 4.15 Hz, H-*o*, Phe-1), 6.77–6.72 (m, 4H, H-*o*, Phe-2 and Phe-3), 6.42 (brs, 1H, NH, Phe), 4.74–4.69 (m, 1H, H-α, Val), 4.65–4.59 (m, 1H, H-α, Phe-1), 4.40 (t, 1H, $J = 4.85$ Hz, H-α, Phe-2), 4.27 (t, 1H, $J = 4.9$ Hz, H-α, Phe-3), 3.58 (3H, s, OCH_3), 3.14–3.06 (m, 6H, H-*β*, Phe-1, Phe-2 and Phe-3), 3.07 (s, 3H, NCH_3, Phe-1), 3.02 (s, 3H, NCH_3, Phe-3), 2.09–2.03 (m, 1H, H-*β*, Val), 1.54 (9H, s, butyl-*t*), 1.02 (6H, d, $J = 4.55$ Hz, H-*γ*, Val) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 175.82$ (C=O, ester), 175.34 (C=O, Phe-2), 174.42 (C=O, Val), 172.03 (C=O, Phe-1), 153.51 (C=O, boc), 138.20, 135.28, 133.02 (3C, C-*γ*, Phe-3, Phe-2 and Phe-1), 130.41, 129.53 (4C, C-*m*, Phe-1 and Phe-2), 130.13, 129.48, 128.29 (6C, C-*o*, Phe-1, Phe-2 and Phe-3), 128.04, 127.72 (2C, C-*p*, Phe-1 and Phe-2), 127.21, 126.04 (3C, C-*m* and C-*p*, Phe-3), 79.63 (C-α, boc), 61.11, 59.23 (2C, C-α, Phe-2 and Phe-3), 53.80 (OCH_3), 51.42, 48.14 (2C, C-α, Phe-1 and Val), 38.03, 35.33, 33.72 (3C, C-*β*, Phe-1, Phe-2 and Phe-3), 32.20 (NCH_3, Phe-1), 29.63 (NCH_3, Phe-3), 28.79 (C-*β*, Val), 29.02 (3C, C-*β*, boc), 18.58 (2C, C-*γ*, Val) ppm.

Synthesis of hirsutide: cyclo (L-Phe-L-N-(Me)Phe-L-Val-L-N-(Me)Phe) (**2**, $C_{34}H_{40}N_4O_4$)

To synthesize cyclopeptide **2**, 3.5 g of linear tetrapeptide unit **1** (5 mmol) was deprotected at the carboxyl end using 0.18 g of LiOH (7.5 mmol) to get Boc-L-Phe-L-*N*-(Me)Phe-L-Val-L-*N*-(Me)Phe-OH. Now, 3.43 g of deprotected tetrapeptide unit (5 mmol) was dissolved in 50 cm^3 of $CHCl_3$ at 0 °C. To this solution, 0.94 g/1.23 g of pnp/pfp (6.7 mmol) and 1.06 g of DCC (5 mmol) were added, and stirring was done at RT for 12 h. The reaction mixture was filtered, and the filtrate was washed with 10% $NaHCO_3$ solution (3 × 15 cm^3) and finally washed with 5% HCl (2 × 10 cm^3) to get the corresponding *p*-nitrophenyl/pentafluorophenyl ester Boc-L-Phe-L-*N*-(Me)Phe-L-Val-L-*N*-(Me)Phe-Opnp/Boc-L-Phe-L-*N*-(Me)Phe-L-Val-L-*N*-(Me)Phe-Opfp. To 3.23 g/3.41 g of this compound (4 mmol) dissolved in 35 cm^3 of $CHCl_3$, 0.91 g of TFA (8 mmol) was added, stirred at RT for 1 h, and washed with two proportions each 25 cm^3 of 10% $NaHCO_3$ solution. The organic layer was dried over anhydrous Na_2SO_4 to get L-Phe-L-*N*-(Me)Phe-L-Val-L-*N*-(Me)Phe-Opnp/L-Phe-L-*N*-(Me)Phe-L-Val-L-*N*-(Me)Phe-Opfp which was dissolved in 25 cm^3 of $CHCl_3$ and 2.8 cm^3/2.21 cm^3/1.61 cm^3 of TEA/NMM/pyridine (21 mmol) was added. Then, whole contents were kept at 0 °C for 7 days. The reaction mixture was washed with 10% $NaHCO_3$ (3 × 25 cm^3) and 5% HCl (3 × 15 cm^3) solutions. The organic layer was dried over anhydrous Na_2SO_4, and the crude cyclized product was crystallized from $CHCl_3$/*n*-hexane to get 2.3 g (81%, NMM), 1.95 g (67%, C_5H_5N), 1.5 g (53%, TEA) of pure **2** as white solid. M.p.: 202 °C; R_f 0.61 ($CHCl_3$:AcOH:H_2O/3:2:5); $[\alpha]_D$ -192.2×10^{-1} deg cm^2 g^{-1} (Ref. [13]: $[\alpha]_D$ for natural hirsutide: -192.0×10^{-1} deg cm^2 g^{-1}) (*c*, 0.2 in CH_2Cl_2); IR (KBr): $\bar{\nu} = 3{,}225$–$3{,}216$, 3,110 (N–H str, amide), 2,966, 2,927–2,922 (C–H str, asym, CH_3 and CH_2), 2,875–2,869, 2,845 (C–H str, sym, CH_3 and CH_2), 1,668–1,662, 1,642–1,638 (C=O str, amide), 1,588–1,582, 1,476–1,472 (skeletal bands, rings), 1,539–1,532 (N–H def, amide), 1,378, 1,365 (C–H bend, propyl-*i*), 717–706, 697–

Table 1 Antibacterial activity data

Compd	Zone of inhibition/mm			
	Bacterial strains			
	C. pyogenes	*S. aureus*	*P. aeruginosa*	*K. pneumoniae*
1	8 (25)	9 (13)	19 (6)	21 (6)
2	10 (25)	11 (13)	28 (6)	26 (6)
Control	–	–	–	–
Std1	20 (13)	28 (6)	24 (6)	25 (6)

Values in brackets are MIC values (μg cm^{-3})

Std1 Gatifloxacin

Table 2 Antifungal activity data

Compd	Zone of inhibition/mm			
	Fungal strains			
	C. albicans	*M. audouinii*	*A. niger*	*Ganoderma* sp.
1	8 (13)	10 (6)	8 (25)	16 (6)
2	9 (13)	14 (6)	11 (25)	20 (6)
Control	–	–	–	–
Std2	20 (6)	17 (6)	18 (13)	22 (6)

Values in brackets are MIC values (μg cm^{-3})

Std2 Griseofulvin

686 (C–H bend, oop, rings) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 9.58$ (brs, 1H, NH, Phe), 9.45 (brs, 1H, NH, Val), 7.27–7.22 (m, 4H, H-*m*, Phe-2 and Phe-3), 7.15 (tt, 2H, $J = 7.15$, 4.35 Hz, H-*m*, Phe-1), 7.08–7.02 (m, 2H, H-*p*, Phe-2 and Phe-3), 6.98 (t, 1H, $J = 6.15$ Hz, H-*p*, Phe-1), 6.87 (dd, 2H, $J = 8.75$, 4.15 Hz, H-*o*, Phe-1), 6.75–6.69 (m, 4H, H-*o*, Phe-2 and Phe-3), 5.75–5.70 (m, 1H, H-α, Val), 5.35–5.31 (m, 1H, H-α, Phe-1), 5.25–5.21 (m, 1H, H-α, Phe-2), 5.20–5.16 (m, 1H, H-α, Phe-3), 2.91 (s, 3H, NCH_3, Phe-1), 2.87 (s, 3H, NCH_3, Phe-3), 2.69–2.58 (m, 6H, H-β, Phe-1, Phe-2 and Phe-3), 1.65–1.58 (m, 1H, H-β, Val), 1.15 (6H, d, $J = 4.6$ Hz, H-γ, Val); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 174.53$ (C=O, Phe-1), 174.14 (C=O, Val), 172.20 (C=O, Phe-3), 169.91 (C=O, Phe-2), 139.02, 137.68 (3C, C-γ, Phe-2, Phe-3 and Phe-1), 131.18 (2C, C-*o*, Phe-1), 130.59, 129.76 (4C, C-*m*, Phe-2 and Phe-3), 129.23 (2C, C-*m*, Phe-1), 128.74, 128.21 (4C, C-*o*, Phe-2 and Phe-3), 127.90 (2C, C-*p*, Phe-2 and Phe-3), 127.21 (C-*p*, Phe-1), 63.83, 60.22 (2C, C-α, Phe-2 and Phe-3), 57.50, 55.87 (2C, C-α, Val and Phe-1), 45.13, 43.64, 40.18 (3C, C-β, Phe-1, Phe-2 and Phe-3), 34.59 (N*C*H_3, Phe-1), 32.36 (N*C*H_3, Phe-3), 31.66 (C-β, Val), 18.73 (2C, C-γ, Val) ppm; FABMS (70 eV): $m/z = 569.70$ [$(M + 1)^+$, 100], 541.70 [(569.70–CO)$^+$, 23], 470.60 [(*N*(Me)Phe-Phe-*N*(Me)Phe)$^+$, 41], 442.60 [(470.60–CO)$^+$, 11], 422.60 [(*N*(Me)Phe-Val-*N*(Me)Phe)$^+$, 16], 408.50 [(Phe-*N*(Me)Phe-Val)$^+$/(Val-*N*(Me)Phe-Phe)$^+$, 29], 394.50 [(422.60–CO)$^+$, 37], 380.50 [(408.50–CO)$^+$, 63], 309.40 [(*N*(Me)Phe-Phe)$^+$/(*N*(Me)Phe-Phe)$^+$, 19], 281.40 [(309.40–CO)$^+$, 58], 261.40 [(*N*(Me)Phe-Val)$^+$/(Val-*N*(Me)Phe)$^+$, 11], 233.30 [(261.40–CO)$^+$, 17], 162.20 [(*N*(Me)Phe)$^+$, 69], 148.20 [(Phe)$^+$, 13], 134.20 [$(C_9H_{12}N)^+$/(162.20–CO)$^+$, 34], 120.20 [$(C_8H_{10}N)^+$, 8], 100.20 [(Val)$^+$, 11], 91.10 [$(C_7H_7)^+$, 17], 72.10 [$(C_4H_{10}N)^+$, 21], 65.10 [$(C_5H_5)^+$, 14], 43.10 [$(C_3H_7)^+$, 6], 42.10 [$(C_3H_6)^+$, 4], 15.10 [$(CH_3)^+$, 3].

Pharmacological activities

The newly synthesized linear as well as cyclic tetrapeptides **1** and **2** were screened for antimicrobial activity [29]

Table 3 Antihelmintic activity data

Compd	Conc./mg	Earthworm species					
		M. konkanensis		*P. corethruses*		*Eudrilus* sp.	
		Mpt/min	Mdt/min	Mpt/min	Mdt/min	Mpt/min	Mdt/min
1	100	10.28 (±0.36)	19.54 (±0.23)	12.46 (±0.16)	23.09 (±0.47)	15.22 (±0.51)	25.38 (±0.23)
	200	06.44 (±0.50)	13.27 (±0.22)	07.50 (±0.20)	17.13 (±0.36)	10.29 (±0.41)	18.12 (±0.49)
2	100	07.44 (±0.43)	14.52 (±0.21)	09.28 (±0.33)	19.04 (±0.28)	12.40 (±0.50)	23.45 (±0.31)
	200	04.09 (±0.30)	10.41 (±0.17)	05.34 (±0.61)	12.33 (±0.43)	09.42 (±0.31)	16.55 (±0.66)
Std3	100	11.25 (±0.18)	19.44 (±0.40)	13.20 (±0.54)	24.22 (±0.11)	12.32 (±0.33)	23.35 (±0.42)
	200	06.40 (±0.37)	13.12 (±0.83)	08.60 (±0.50)	18.04 (±0.19)	07.37 (±0.28)	16.11 (±0.21)
Std4	100	13.85 (±0.64)	22.90 (±0.53)	17.85 (±0.48)	29.64 (±0.73)	13.54 (±0.45)	24.05 (±0.62)
	200	08.21 (±0.48)	15.11 (±0.28)	12.44 (±0.21)	21.05 (±0.39)	10.18 (±0.77)	18.52 (±0.30)
Control	–	–	–	–	–	–	–

All data are given as mean ± SD ($n = 3$)

Mpt Mean paralyzing time, *Mdt* mean death time, *Std3* albendazole, *Std4* mebendazole

Table 4 Cytotoxic activity data

Compd	Conc./μg cm^{-3}	DLA cells			EAC cells		
		Dead cells	%GI	IC_{50}	Dead cells	%GI	IC_{50}
1	62.50	38	100.00		28	100.00	
	31.25	31	81.58		22	78.57	
	15.63	20	52.63	21	13	46.43	25
	07.81	11	28.95		03	10.71	
	03.91	07	18.42		01	03.57	
2	62.50	38	100.00		28	100.00	
	31.25	34	89.47		24	92.86	
	15.63	25	65.79	14	16	68.42	22
	07.81	19	50.00		06	21.43	
	03.91	12	31.58		03	10.71	
Control	62.50	00	–		00	–	
	31.25	00	–		00	–	
	15.63	00	–	–	00	–	–
	07.81	00	–		00	–	
	03.91	00	–		00	–	
Std5	62.50	38	100.00		28	100.00	
	31.25	38	100.00		28	100.00	
	15.63	28	73.68	37	17	60.71	91
	07.81	25	65.79		09	32.14	
	03.91	16	42.11		05	17.86	

% Growth inhibition (%GI) = 100 – [{($Cell_{total}$ – $Cell_{dead}$) × 100}/$Cell_{total}$], IC_{50} cytotoxic concentration inhibiting 50% of percentage growth (in μM)

Std5 5-fluorouracil (5-FU)

against the four bacterial strains *Corynebacterium pyogenes* (MUMC 73), *Staphylococcus aureus* (MUMC 377), *Pseudomonas aeruginosa* (MUMC 266) and *Klebsiella pneumoniae* (MUMC 95) and four fungal strains *Microsporum audouinii* (MUMC 545), *Candida albicans* (MUMC 29), and *Aspergillus niger* (MUMC 77) and *Ganoderma* sp. (MUMC 218) at 25–6 μg cm^{-3} using gatifloxacin and griseofulvin as standard drugs. MIC values of the test compound were determined by tube dilution technique using sterile DMF. The petri plates inoculated with bacterial/fungal cultures were incubated at 37 °C for 18 and 48 h. The average diameters of the zones of inhibition (in mm) of the test compound were calculated for triplicate sets and compared with that produced by the standard drugs.

Antihelmintic activity studies [30] were carried out against the three different species of earthworms *Megascoplex konkanensis* (ICARBC 211), *Pontoscotex corethruses* (ICARBC 117), and *Eudrilus* sp. (ICARBC 042) at 1 and 2 mg cm^{-3} concentration using albendazole and mebendazole as reference drugs. The mean paralyzing and death times were calculated for triplicate sets. The death time was ascertained by placing the earthworms in warm water (50 °C), which stimulated movement if the worm was alive.

Short-term in vitro cytotoxicity studies [31] were performed against *Dalton's lymphoma ascites* (NCRC 101) and *Ehrlich's ascites carcinoma* (NCRC 69) cell lines at 62.5–3.91 μg cm^{-3} using 5-fluorouracil (5-FU) as reference compound. Activity was assessed by determining the percentage inhibition of DLA and EAC cells. IC_{50} values were determined by the graphical extrapolation method.

Detailed procedures of pharmacological activity studies are mentioned in our previously published reports [32]. The results of biological activity studies are compiled in Tables 1, 2, 3 and 4.

Acknowledgments We are grateful to USIC, DU, Delhi, India, and RSIC, IIT, Delhi, India, for spectral analysis and CPCRI, Kasaragod, Kerala, India, for providing earthworms for testing the antihelmintic activity. Also, great thanks to JSS College of Pharmacy, Ooty, India, for cytotoxic activity studies.

References

1. Capon RJ, Ratnayake R, Stewart M, Lacey E, Tennant S, Gill JH (2005) Org Biomol Chem 3:123
2. Ravindra G, Ranganayaki RS, Raghothama S, Srinivasan MC, Gilardi RD, Karle IL, Balaram P (2004) Chem Biodivers 1:489
3. Dahiya R, Pathak D (2006) Egypt Pharm J (NRC) 5:189
4. Sun Y, Tian L, Huang YF, Sha Y, Pei YH (2006) Pharmazie 61:809
5. Tan LT, Cheng XC, Jensen PR, Fenical W (2003) J Org Chem 68:8767
6. Thongtan J, Saenboonrueng J, Rachtawee P, Isaka M (2006) J Nat Prod 59:713
7. Baute R, Deffieux G, Merlet D, Baute MA, Neveu A (1981) J Antibiot (Tokyo) 34:1261

8. Oh DC, Kauffman CA, Jensen PR, Fenical W (2007) J Nat Prod 70:515
9. Strobel GA, Miller RV, Martinez-Miller C, Condron MM, Teplow DB, Hess WM (1999) Microbiology 145:1919
10. Adachi K, Kanoh K, Wisespongp P, Nishijima M, Shizuri Y (2005) J Antibiot (Tokyo) 58:145
11. Vongvanich N, Kittakoop P, Isaka M, Trakulnaleamsai S, Vimuttipong S, Tanticharoen M, Thebtaranonth Y (2002) J Nat Prod 65:1346
12. Seto Y, Takahashi K, Matsuura H, Kogami Y, Yada H, Yoshihara T, Nabeta K (2007) Biosci Biotechnol Biochem 71:1470
13. Lang G, Blunt JW, Cummings NJ, Cole ALJ, Munro MHG (2005) J Nat Prod 68:1303
14. Dahiya R, Pathak D, Himaja M, Bhatt S (2006) Acta Pharm 56:399
15. Dahiya R (2007) J Chil Chem Soc 52:1224
16. Dahiya R, Sharma RD (2008) Eur J Sci Res 21:277
17. Dahiya R (2008) Chem Pap 62:527
18. Das P, Lal VK, Saxena V, Himaja M (2007) Indian J Hetrocycl Chem 17:71
19. Dahiya R (2007) Pak J Pharm Sci 20:317
20. Dahiya R (2007) Acta Pol Pharm 64:509
21. Dahiya R, Kaur K (2007) Arch Pharm Res 30:1380
22. Dahiya R, Pathak D (2007) J Serb Chem Soc 72:101
23. Dahiya R (2008) Turk J Chem 32:205
24. Dahiya R (2008) Arch Pharm 341:502
25. Dahiya R (2008) J Iran Chem Soc 5:445
26. Dahiya R, Kumar A (2008) J Zhejiang Univ Sci B 9:391
27. Dahiya R, Kaur K (2008) Arzneimittelforschung 58:29
28. Dahiya R, Bansai Y (2008) Res J Chem Environ 12:52
29. Bauer AW, Kirby WM, Sherris JC, Turck M (1966) Am J Clin Path 45:493
30. Garg LC, Atal CK (1963) Indian J Pharm Sci 59:240
31. Kuttan R, Bhanumathy P, Nirmala K, George MC (1985) Cancer Lett 29:197
32. Dahiya R, Pathak D (2007) Eur J Med Chem 42:772